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STAIN TECHNOLOGY

VOLUME III JULY, 1928 NUMBER 3

CULTURAL SEPARATION OF BACTERIA ON THE BASIS OF TRIPHENYL METHANE CO-EFFICIENTS

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ABSTRACT.—**The general parallelism between the Gram reaction and *normal* selective bacteriostasis by the triphenylmethane dyes is well established, as is also the existence of a small number of organisms in each group which do not follow the rule. *Reverse* extrinsic bacteriostasis has been demonstrated, but only within a very limited field. The discovery of substances possessing reverse selective power, comparable in extent to normal selective power, would be of value. In the absence of such substances, the slight quantitative differences in the behavior toward dyes, of organisms belonging to the same Gram group, may be turned to account. The authors have determined with great accuracy the crystal violet coefficients of five Gram-positive and five Gram-negative organisms. These observations show that all known aerobic organisms could probably, on the basis of their triphenylmethane coefficients, be placed on a curve, which would on the whole, parallel the Gram reaction. The possibilities of separating Gram-negative organisms from Gram-positives by means of the dyes are well understood. The authors cite experiments to show that similar separations may be made within the Gram groups by making use of the *quantitative* differences in triphenylmethane coefficients.**

In the original survey of the bacterial field, as to the nature of the bacteriostatic properties of the triphenylmethane dyes, which resulted, after an examination of 130 species in the establishment of the principle of parallelism between Gram reaction and selective bacteriostasis, it was shown that 90% of both Gram-positive and Gram-negative organisms followed the rule and that about 10% of exceptions existed in both groups. It was also definitely stated that "the bacteriostatic action of gentian violet is, like the action of all bactericides, fundamentally a quantitative one; that is to say, when

the dye is applied in sufficient intensity and for a sufficiently long time, it will probably inhibit or kill all organisms. The interval, however, between the dilution necessary to prevent the growth of *B. subtilis* and that required to have an appreciable effect on *B. prodigiosus*, is so wide that the selective action may almost be thought of as independent of any quantitative element."[1]

The large amount of work done in this field since 1912 has entirely confirmed the original conclusions. Sufficient emphasis was laid in the original communication on the exceptional organisms, to make it unnecessary in subsequent publications always to refer to the exceptions when the much larger range of the parallelism was under discussion. On the basis of this omission certain observers have criticized the statement as to the parallelism between the Gram reaction and selective bacteriostasis. Thus Stearn and Stearn[2] wrote, "It is misleading to state, as certain observers do, (Churchman 1923) that the Gram-positive organisms are selectively inhibited by these dyes" exactly as if the 1923 publication had not been preceded by that of 1912, in which the very point these authors were making had been established.

Altho there is reason for believing that acid fuchsin under certain very limited conditions is capable of exhibiting a "reverse extrinsic bacteriostasis"—(its intrinsic selective power follows the rule)[3] no dye is known which possesses a reverse selective power at all comparable to the normal selective power of the triphenylmethanes. The discovery of such a property in a dye or other substance would undoubtedly prove most useful since we would then be able to inhibit selectively either the Gram-negative or the Gram-positive groups at will.

It is the purpose of the present communication to show that in the absence of substances possessing such reverse selective powers, the slight quantitative differences in behavior toward gentian violet exhibited by organisms belonging to the same Gram group, may well be turned to account. The instances which will be cited of this use of triphenylmethane dyes indicate that the possibilities have by no means been exhausted. Selective bacteriostasis on the basis of difference in gentian violet coefficient within the Gram-negative group,

---

[1]Churchman, J. W. Selective Bactericidal Action of Gentian Violet. J. Exp. Med. **16**, 221–247; at 237. 1912.

[2]Stearn, A. E., and E. W. The Chemical Mechanism of Bacterial Behavior. J. Bact. **9**, 491–510. 1924.

[3]This complex subject is discussed by Churchman, J. W. The Mechanism of Selective Bacteriostasis. Proc. Nat. Acad. Sci. **9**, 78. 1923.

has already been reported for *B. enteritidis* and *B. coli*;[4] and Hall[5] has shown the possibilities of freeing Gram-positive anaerobes from Gram-positive facultative aerobic contamination by this method.

Altho it was clearly recognized in the original experiments on the selective powers of gentian violet, that the reaction was fundamentally a quantitative one, only rough methods were used in the studies made at that time, since they perfectly served the purposes then in view. It has recently seemed of importance to review the bacterial field, and to determine with great accuracy the amount of dye needed to inhibit the growth of all the better known organisms. To begin this study, the crystal violet coefficient has been determined for ten organisms, two Gram-positive spore-bearers, three Gram-positive non-spore-bearers, and five Gram-negatives. The organisms used were obtained from various sources and are here denoted by the following names:

NAME	SOURCE
Bacillus anthracis Koch	Amer. Type Culture Collection No. 10
B. subtilis Cohn	Isolated from hay infusion in this laboratory[6]
B. diphtheriae Klebs	New York Board of Health
Staphylococcus aureus Rosenbach	Isolated by Torrey from a septicaemic patient
Rhodococcus roseus (Flügge) Holland	Amer. Type Culture Collection No. 144
B. coli communis (Escherich) Sternberg	Isolated by Torrey from human feces
B. typhosus Zopf	New York Board of Health
B. dysenteriae Shiga	Amer. Type Culture Collection No. 51
B. proteus vulgaris (Hauser)	Amer. Type Culture Collection No. 93
Bacterium pyocyaneum Lehm. & Neum	Amer. Type Culture Collection No. 97

A complete description of the technic employed will appear in a later publication. It may here be stated that the most accurate methods possible were used thruout. Every controllable source of error was avoided. The figures given in the chart may therefore be regarded as correctly indicating the crystal violet coefficient of these organisms *under the conditions of the experiment.*[7] Absolutely consistent results were obtained with all the organisms save *B. proteus vulgaris*, and with this organism the variations were not great. In

[4]Churchman, J. W. and Michael, W. H. Selective Action of Gentian Violet on Closely Related Bacterial Strains. J. Exp. Med. **16,** 822–830. 1912.

Churchman, J. W. Further Studies on the Behavior of Bacteria Toward Gentian Violet. J. Exp. Med. **33,** 569–581. 1921.

[5]Hall, I. C. Selective Elimination of Hay Bacillus from Cultures of Obligative Anaerobes. J. A. M. A. **72,** 274–275. 1919.

Hall, I. C. Practical Methods in the Purification of Obligate Anaerobes. J. Inf. Dis. **27,** 576–590. 1920.

[6]The identification of this culture was based upon a comparison with the description given in Bergey's Manual of Determinative Bacteriology.

[7]That is when plated out in plain agar. The coefficients are different when tested by surface plants in plain agar, or in liquid medium, or in the presence of serum, etc.

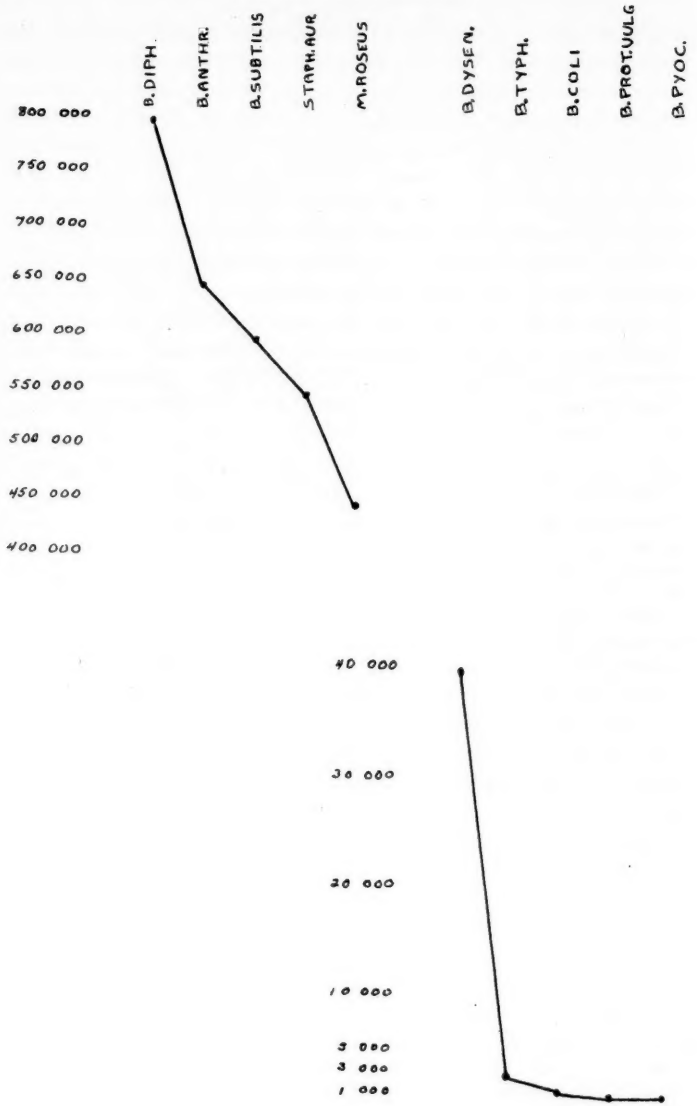

Chart 1. A chart showing the curves for the gentian violet coefficients of 10 organisms, 5 Gram-positive and 5 Gram-negative. The graph on the left represents the curve for the Gram-positive group, that on the right the curve for the Gram-negative. For economy of space the two graphs have been drawn to different scale. The chart illustrates the fact that bacteria may be arranged in a series on the basis of triphenylmethane coefficients, that a sharp demarcation exists between the majority of the Gram-positives and the majority of the Gram-negatives, but that gradations also exist within each Gram group.

the chart, the curves for the Gram-positive and Gram-negative groups, have had, for reasons of space, to be drawn to different scale. It is clear that the members of each group form a series, that between the two groups there is a great gap, (1–450,000 to 1–40,000), and that the most resistant Gram-negative organism (*B. pyocyaneus*) is 800 times as resistant to the bacteriostatic power of this dye as the least resistant Gram-positive organism (*B. diptheriae*). If the scope of this survey were extended, all the known aerobic organisms would probably fall into line. The Gram-positives would tend to fall in the

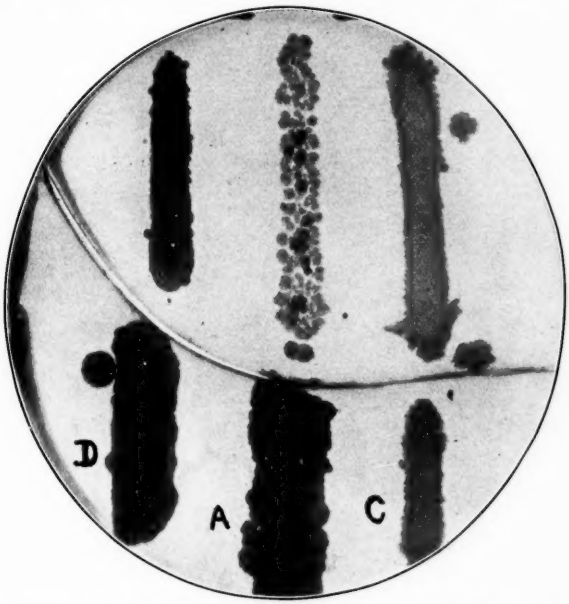

Fig. 1. Lower half of divided plate contains plain agar, upper half contains crystal violet agar. 1–5,000,000. (D) *B. dysenteriae* (Shiga), (A) *B. anthracis*, and (C) *B. coli communis*, Cf with Figs. 2 and 3.

upper range, the Gram-negatives in the lower, and there would doubtless be a middle range where the relationship would be contradictory. It might well be that if the bacterial field could be completely covered, a continuous curve could be constructed representing progressive crystal violet coefficients. On the whole, such a curve would parallel the Gram reaction, but there would be points of marked variation from it.

The variations of crystal violet coefficient *within each of the two Gram groups*, while much less striking than the variations between the

two groups, are sufficient to be, perhaps, of some practical value. Browning,[8] in referring to my statement that the selective powers of the triphenylmethanes might prove of value, rather deprecated the idea in the following terms: "Churchman emphasized the possibilities thus advanced for the isolation of the violet resistant organisms from mixtures with susceptible types, but since in most instances, the difficulties in separation lie in the opposite direction, *i. e.*, the Gram-negative bacilli tend to overgrow the others, this fact is of no great practical importance, except for the isolation of the tubercle bacilli."

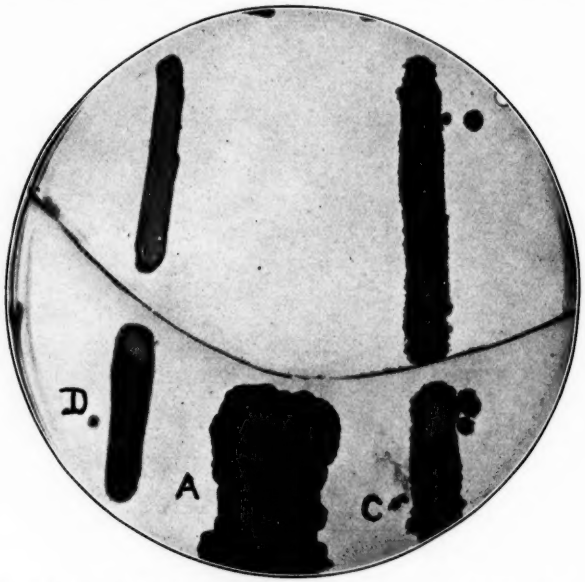

Fig. 2. Upper half of plate contains crystal violet agar. 1–40,000. Cultures used are the same as in Figs. 1 and 3.

This statement of the case, while correct so far as it goes, overlooks certain possibilities in this field. In the experiments from which the chart here reproduced, was constructed, the growth of *B. coli* was unaffected by 1–40,000 of the dye. At this dilution the growth of *B. dysenteriae* was completely inhibited. Theoretically, therefore, it should be possible to separate these two organisms on the basis of their crystal violet coefficients. Records of an experiment which showed the possibilities of this kind of cultural separation are pre-

[8]Browning, C. H. Applied Bacteriology. Henry Frowde, Hodder and Stoughton, London, 1918. See p. 89.

sented in Figs. 1, 2, and 3. The divided plate shown in Fig. 1 contained in its upper half, crystal violet 1–5,000,000; the growth of *B. dysenteriae* and of *B. coli* is unaffected; *B. anthracis* grows well, altho even this minute amount of dye has caused some inhibition. The plate shown in Fig. 2 contained dye in a strength of 1–40,000; growth of *B. anthracis* has been entirely prevented; growth of the other two organisms has been unaffected. The plate shown in Fig. 3 contains dye in a strength of 1–20,000;[9] the growth of *B. dysenteriae* has now also been inhibited, but *B. coli* is still unaffected.

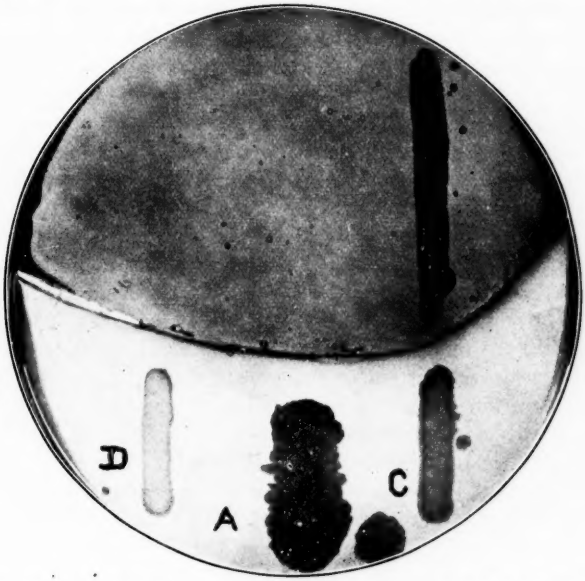

Fig. 3. Upper half of plate contains crystal violet agar. 1–20,000. Cultures used the same as in Figs. 1 and 2.

The faint outline may be seen of the heavy inoculation of *B. dysenteriae* which has been made on the crystal violet agar but no growth has occurred.

A practical opportunity to test out this method of cultural separation of two organisms which fell in the same Gram group, occurred not long ago, and the separation was effected simply by planting the two organisms on media containing dye in the proper strength. A large industrial organization acquired the rights to a method for the pro-

[9]In the experiment illustrated, the inoculations were made on the surface of agar; in the experiments from which the chart was constructed, poured plates were made. Hence the *absolute* values of the crystal violet coefficients are different in the two experiments; the *relative* values remain, however, the same.

duction of a widely used chemical agent in which a fermenting organism was employed. Unfortunately, a contaminant was present in the culture to be used for the fermentation, which could not, during several weeks of study at the industrial laboratory, be removed by ordinary cultural methods. The conclusion had been reached that it was a case of true symbiosis. The problem was referred to this laboratory. Altho both organisms present were Gram-positive, it seemed possible that their triphenylmethane coefficients might be different, and that separation might be effected on this basis. This proved to be indeed the case. The organism desired was more resistant to gentian violet than the contaminant, and by simply trying out a few dilutions of the dye, an appropriate one was reached, at which the desired organism grew unimpeded and the contaminant was completely inhibited. A pure culture resulted.

A table of dye coefficients, based on accurate determinations of the resistance of all organisms to triphenylmethane dyes, would be of value. At least it would appear to be of importance in future to determine the coefficients for all new species. If one could determine the reaction of bacteria to *dye alone*, important data might be at hand for arranging bacteria on the basis of this reaction, and so arriving at further conceptions of their chemical relations to each other. Unfortunately, the many other factors, (such as H-ion concentration, optimum temperature, and carbohydrate needs), which enter into cultural experiments, and which are hardly the same for any two organisms, make it impossible to judge from this kind of experiment, how much of the bacteriostatic effect obtained is due to dye, and how much to unsuitable media. To compare, for example, the susceptibility of *B. diptheriae* to gentian violet, with that of *B. anthracis*, on the basis of experiments done with plain agar, is to overlook the part played in the result by the relative unsuitability of this medium for the former organism.

THE EFFECT OF THE CHEMICAL NATURE OF A DECOLORIZER ON ITS FUNCTIONING.

I. THE GRAM CLASSIFICATION

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ABSTRACT.—**Decolorizers which are distinctly acidic or basic in their chemical nature give abnormally high decolorization in the Gram stain for bacteria. Acidic substances yield more regular results. Ideally an "inert" decolorizer should be used, but ordinarily such substances will not dissolve the dye or dye-mordant precipitate from the smear. The most practical substances seem to be those so very slightly acidic in character as to be practically inert, such as acetone or alcohol, or a mixture of such substances.**

In all the work on differential staining methods comparatively little attention has been paid to systematic observations on decolorizers.

The authors have shown (Stearn and Stearn, 1925) that, by proper buffering, any organism may be made to retain strongly either acidic or basic dye. For example, at high pH-values organisms have increasing tendency to retain basic dye[1] while at low pH-values acid dyes are increasingly retained. Even Gram-negative organisms can be made to retain basic dye strongly if the pH-value is sufficiently high.

Later it was shown (Stearn and Stearn, 1928) that if stained smears were made of either the Gram-positive organism *Bacillus cereus* or the Gram-negative organism *Bacterium coli*, and were buffered in such a manner that in the one case the organisms both retained the basic dye gentian violet and in the other case the acid dye acid fuchsin, (as shown by failure of acetone to produce decolorization) then acidic decolorizers such as phenol, lysol, aldehyde, etc., showed a selective tendency to decolorize the smears stained with basic dye, while basic decolorizers, such as anilin, showed a selective tendency to

[1]A state of confusion in the literature which seems to be increasing should be pointed out. The term "basic dye," or rather the descriptive words "basic" or "acidic" used in connection with dyes or other chemical substances bear no relation to solution reaction or pH. The term basic dye refers to a dye which forms salts with acids. Also an acid dye is one which forms salts with bases. Ordinarily only the dye salts are soluble to any great extent, and they usually hydrolyze, so that the chloride of a basic dye yields an aqueous solution whose reaction is acid, while the alkali salt of an acid dye yields an aqueous solution of alkaline reaction due to hydrolysis.

decolorize smears stained with acid dye. These results could not be explained as due to selective dye solubility, since certain liquids which were less effective as dye solvents were more effective as decolorizers. Neither could they be explained as due to a shift in pH, for either an unbuffered aqueous solution of solid decolorizer or else the organic liquid itself was used.

There seemed to be a chemical factor of reaction between basic decolorizer and acid dye, and vice versa.

In the Gram stain another factor is met with. Here a basic dye is used exclusively for staining. If the mechanism of the reaction is chemical, the bacteria must act in an acidic capacity. Thus basic decolorizers might, by reason of a mass effect due to their large excess over the dye in the smear, displace that dye from combination with the bacterial protein. One would then expect to meet with abnormally great decolorization from acidic decolorizers due to direct combination with the basic dye, and also from basic decolorizers due to replacing the basic dye in its combination with the acidic tissue.

In the following work the effect of decolorizers of different chemical natures on the Gram reaction of representative organisms which are either definitely Gram-positive or Gram-negative was studied. Five cultures were used, including the typhoid and colon organisms, the Staphylococcus, and two Gram-positive spore-formers. The names of the cultures and their sources are:

NAME	SOURCE
Bacillus cereus Frankland	American Type Culture Collection
Bacillus typhosus Zopf; (Rawlings Strain)	Mo. Public Health Laboratory
Bacillus subtilis (Ehrenberg) Cohn	American Type Culture Collection
Bacterium coli (Escherich) Lehmann and Neumann	Isolated from water by Pasadena Public Health Laboratory
Staphylococcus aureus Rosenbach	Isolated from Tumor in Pasadena Hospital

Table 1 gives the results of a study of several decolorizers. Two smears each of the five organisms listed were made on one slide. The preparation was stained for three minutes with an ammonium oxalate solution of gentian violet, mordanted for three minutes with Lugol's iodine solution and then decolorized with the liquid named until the liquid drained free from dye. One-half of the slide was then counter-stained with basic fuchsin. In the table, the upper reading gives the results when no counter-stain was used, the other giving results of the counter-stained preparation.

As will be seen, the acidic substances seem rather regular in their action. The very weakly acidic give in a satisfactory manner the accepted differentiation. The change with more distinct acidic properties is invariably toward increased decolorization. The more

distinctly acidic tend to give complete decolorization in all cases. This is in accord with the findings of Kisskalt (1901) who showed, using a series of 39 organisms, that each member of the series of alcohols, amyl, propyl, butyl, ethyl and methyl, decolorized fewer strains than the one next following. The order is that of increasing acidic strength.

TABLE 1. SHOWING THE VARYING EFFECT OF DIFFERENT DECOLORIZERS ON THE GRAM TECHNIC

DECOLORIZER	B. CEREUS	B. TYPHOSUS	B. SUBTILIS	B. COLI	STAPH.
		(Very weakly acidic)			
Acetone	+*	−	+	−	+
	+	−	+	−	+
Methyl alcohol	+	−	+	−	
	+	−	+	−	
		(More distinctly acidic)			
Acetoacetic ester	+	−	+	−	+
	+	−	+	−	+
Phenol	−	−	−	−	−
	−	−	−	−	−
o-Cresol	− − +	−	− − +	−	−
	− − +	−	−	−	−
Lysol	−	−	− − +	−	−
	−	−	− − +	−	−
		(Basic decolorizers)			
Anilin	+ −	−	+	−	+
	+ −	−	+ + −	−	+ + −
Methyl anilin	+	− +	+	− +	+
	+ + −	−	+ + −	− +	+
Di-methyl anilin	+	+ −	+	+ −	+
	+	+ −	+	+ −	+
o-Toluidin	+	−	+	−	+
	+ −	−	+ + −	−	+ + −
Pyridin	+ −	+ −	+	+ −	+
	+ −	−	+ + −	−	+ + −

*The plus signs indicate Gram-positiveness.

The upper result is for a smear not counterstained, while the lower is for a counterstained smear.

The organisms used in the present study are not considered among the Gram-variable. They were selected as being either distinctly Gram-positive or Gram-negative. The effect of such a decolorizer as phenol cannot be due to enormously increased solubility of dye, since the solubility of the dye-mordant precipitate on the slide is very much greater in acetone (as judged by speed of solution). Nor can it be explained as due to a shift in pH, for the decolorizer used was

nearly pure phenol and even a double normal aqueous solution would have a pH of only about 4.5 whereas these Gram-positive organisms retain basic dye to a much lower pH-value when decolorized with acetone. (Stearn and Stearn, 1925.) The obvious explanation seems to be the acidic character of the phenol.

TABLE 2. SHOWING THE VARYING EFFECT OF DIFFERENT DECOLORIZERS ON THE GRAM TECHNIC

Analogous to Table 1 except that smears were mordanted with alkaline iodine solution instead of Lugol's solution

DECOLORIZER	B. CEREUS	B. TYPHOSUS	B. SUBTILIS	B. COLI	STAPH.
		(Very weakly acidic)			
Acetone	+*	—	+	—	+
	+	—	+	—	+
Methyl alcohol	+	—	+	—	+
	+	—	+	—	+
		(More distinctly acidic)			
Phenol	—	—	—	—	—
	—	—	—	—	—
Lysol	—	—	—	—	—
	—	—	—	—	—
		(Basic decolorizers)			
Anilin	+	—	+ (weak)	—	+ —
	+ —	—	— — +	—	— — +
Di-methyl anilin	+ —	— — +	+ —	— +	+
	+ —	—	+ —	—	+
Pyridin	+	—	+	—	+
	+ —	—	+ —	—	+ —

*The plus sign indicates Gram-positiveness.

The upper result is for a smear not counterstained, while the lower is for a counterstained smear.

With the basic decolorizers we might at first thought expect abnormally small decolorizing power. Just as Gram-positive organisms seem negative with certain acidic decolorizers so Gram-negative might be expected to appear positive at times with basic decolorizers. While this is actually the case at times as is seen in the table, it is equally true that the real Gram-positive organisms, instead of appearing more strongly positive as might be expected, may at the same time be partly decolorized. With basic decolorizers in the Gram technic there is thus another factor to be considered. With acidic decolorizers the problem is simple. They tend to combine with basic dye and thus break up the dye-bacterial compound which might be stable in the presence of an inert decolorizer. While the basic decolor-

izer has no tendency to combine with the basic dye, there is no reason to suppose that, if basic dye and bacteria form a semi-stable compound, basic decolorizer and bacteria under the same conditions will not act likewise, with displacement of dye. It is also not unreasonable to suppose that the greater the tendency of bacteria to retain basic dye, the greater also will be the tendency to retain basic decolorizer. Indeed it seemed that in many cases smears decolorized with such decolorizers would show a group of Gram-positive organisms partly decolorized before all the Gram-negative organisms on an adjacent smear were decolorized. This is also brought out in Table 1.

A few slides were run thru the same general technic as outlined above except that the preparations were mordanted with alkaline iodine solution as recommended by Kopeloff and Cohen (1928). Results of this experiment are given in Table 2. Here the action of the acidic decolorizers is about the same as in the previous experiment, but, even tho the mordanting pH-value is now such that the dye-bacterial compound is more stable than when using Lugol's solution, the decolorization with basic agents is for the most part greater. The authors cannot account for such a finding on the basis of any physical theory of mechanism of the Gram reaction.

Results

The results here presented suggest a new factor in differential staining and, it is hoped, suggest a rational basis for a consideration of the problem of decolorization. The importance of the acidic or basic character of a decolorizer does not, of course, decrease the importance of other well recognized factors. The H-ion concentration at which mordanting is carried on is being recognized in later modifications of the Gram technic, and the solubility of dye or dye-mordant precipitate in the decolorizer is a well recognized factor.

An "inert" decolorizer (*i.e.*, from the point of view of acidic or basic properties) would be ideal. Such a one is practically unavailable, however, since insolubility of dye-mordant precipitate precludes the use of any, at least of the common ones. The next most consistent type so far as results are concerned seems to be the very weakly acidic —such substances as are at present being used either pure or in mixture.

In the following paper the symmetry between the action of acidic and basic decolorizers will be pointed out. This cannot be done in studying the Gram technic since only basic dyes are employed for staining and the abnormal effects of basic and acidic decolorizers depend on seemingly different factors. However, when one studies

staining reactions in which H-ion concentration is controlled and both types of dyes are used, such symmetry is easily seen.

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THE EFFECT OF THE CHEMICAL NATURE OF A DECOLORIZER ON ITS FUNCTIONING.

II. THE APPARENT ISOELECTRIC POINT

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ABSTRACT.—**The isoelectric point of a bacterial system is the hydrogen-ion concentration at which there is equal retention of anion and cation. Defining this point as that at which there is equal retention of acidic and basic stain when acetone is used as a decolorizer, it is shown that acidic decolorizers shift the experimentally determined point to a higher pH-value while basic decolorizers shift it to a lower value. Thus basic decolorizers show abnormally high decolorizing power toward smears stained with acid dyes, and acid decolorizers show the same abnormal behavior toward smears stained with basic dye. By basic decolorizer is meant, not one of high pH-value, but one which will form a salt with acids, as for example pyridin or anilin. This indicates an ionic chemical equilibrium as a factor in the mechanism of staining.**

In the preceding paper it was shown that acidic decolorizers regularly exhibit abnormally high decolorizing power toward smears stained with basic dye, and this power seems to increase roughly with the acidic strength of the decolorizer. Basic decolorizers were also shown to exhibit abnormally high decolorizing power in some cases, but no regularity of behavior could be noted. In this paper it is shown that the regularity in the results of acidic decolorizers toward smears stained with basic dye can be found in the case of basic decolorizers when the smears are stained with acid dyes.

As has been shown (Stearn and Stearn, 1924a), organisms are characterized by amphoteric properties. Their isoelectric points may be determined by studying the effect of H-ion concentration on their retaining power for basic and acidic dyes (Stearn and Stearn, 1925). Such an isoelectric condition of a pure water suspension of an organism is a function of pH only and may be determined in a fairly reproducible manner by staining methods, if a decolorizer be used which dissolves excess free dye but which does not tend to decompose any compound between dye and organism. The authors have found acetone best for such a purpose.

In the present paper the effect of substituting distinctly acidic and basic decolorizers for acetone on the apparent isoelectric point of

organisms is described. From a chemical point of view it may be predicted that acidic decolorizers will have a greater tendency to "leach out" basic dye under ordinary conditions than basic decolorizers, and the opposite may be predicted for basic decolorizers, *i.e.* their selective tendency should be exerted toward acid dyes. Thus acidic decolorizers should shift the apparent isoelectric point to a higher pH-value and basic decolorizers should shift it to a lower pH-value than that determined by means of what might be termed a chemically inert decolorizer.

The same five organisms employed in the preceding paper[1] were used in the study, namely the Gram-positive spore-formers *Bacillus cereus*, *Bacillus subtilis* and a strain of Staphylococcus, and the Gram-negative organisms *Bacillus typhosus* and *Bacterium coli*. To insure uniform conditions for all organisms, smears of each were made on the same slide. The slides were stained for two minutes in the one case with gentian violet and in the other with acid fuchsin. The slides were then flooded with a buffer solution adjusted to a definite pH-value and thus buffered for three minutes. Finally they were intensively decolorized, *i.e.* until the decolorizer drained from the slide free from dye. A few typical results for *Bacterium coli* and for Staphylococcus are given in figures 1 and 2. The pH-values noted are in all cases those of the mordanting buffer.

Besides those noted in the figures, other basic and acidic substances were used. In all cases similar behavior was observed. Acidic decolorizers shifted the apparent isoelectric point to a higher pH-value and basic decolorizers shifted it to a lower pH-value than that given by acetone.

The isoelectric point of such a complex system as an organism cannot be defined as the hydrogen-ion concentration at which neither anion nor cation is retained. One would not consider such a definition, even theoretically, except for a single pure ampholyte under certain conditions; and certainly the curves with acetone show that at the isoelectric point a considerable amount of both dye anion and dye cation may be retained. One should preferably define the isoelectric point of such a system as an organism as the hydrogen-ion concentration at which there is equal retention of cation and of anion. For measuring such retention by staining methods the decolorizer should, of course, be one which would itself introduce no ionic complications. It should remove uncombined dye by solution only. Acetone has seemed the nearest approach, among practically usable substances, to an ideal, or chemically inert, decolorizer, and isoelectric

[1]See page 82.

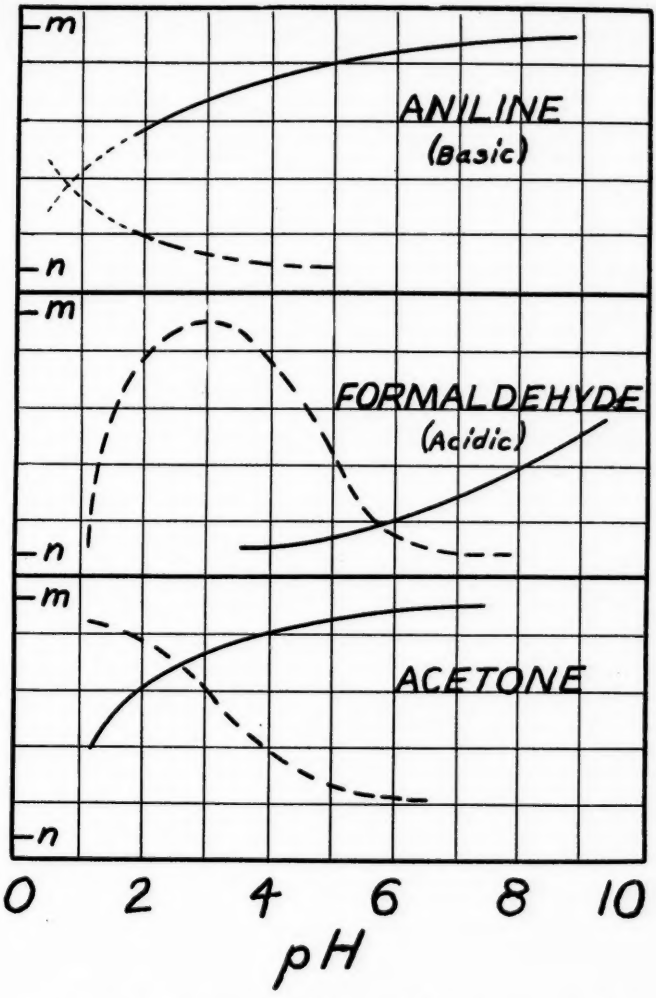

Figure 1. Showing the apparent retaining power of *Staphylococcus aureus* for gentian violet (unbroken lines) and acid fuchsin (broken lines) as a function of pH. Abscissae represent pH-values while ordinates give the intensity of retained color, after decolorization with the decolorizer indicated, based on an arbitrary scale. The scale is between the ordinates *m*, representing maximum retained color, and *n*, representing complete decolorization. The acetone point lies at a pH-value of about 2.6, the formaldehyde point at about 5.8, and the anilin point (obtained by extrapolation) at about 0.8.

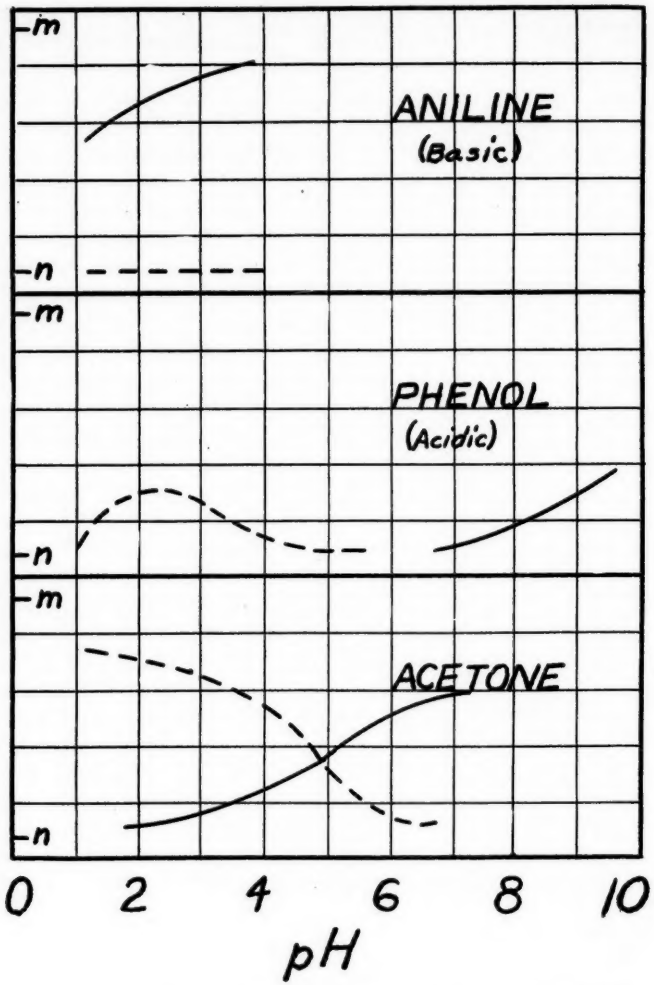

Figure 2. Showing the apparent retaining power of *Bacterium coli* for gentian violet (unbroken lines) and acid fuchsin (broken lines) as a function of pH. Abscissae represent pH-values, while ordinates give the intensity of retained color, after decolorization with the decolorizer indicated, based on an arbitrary scale. The scale lies between the ordinate *m*, representing maximum retained color, and *n*, representing complete decolorization. The acetone point lies at a pH-value of about 5, the phenol point is indeterminate since there was complete decolorization thru the total pH-range between 5 and 7, the anilin point would have to be obtained by extrapolation and seems to lie close to pH 0, or even at a negative pH-value.

points determined, using this decolorizer, are reproducible, so that the isoelectric points of the organism have been more or less arbitrarily defined by the authors in their work as what might be termed the "acetone" isoelectric points.

The results presented in figures 1 and 2 are not to be interpreted as showing actual shifts of the chemical isoelectric points of the organisms. These results are plotted to show that, by using a decolorizer which is not practically chemically inert to both stain and organism, the organism acts as tho it were one of different isoelectric point.

A table might be prepared showing the apparent isoelectric points of the various organisms using different decolorizers; but the values, aside from the acetone points, would have no quantitative significance. Indeed, certain of them can hardly be experimentally realized, but have to be approximated by extrapolation. Reference to the anilin curves in Fig. 2 shows that the apparent isoelectric point of *Bacterium coli* as given using anilin as decolorizer would probably lie at a negative pH-value.

The authors showed some years ago that the Gram character of an organism was directly correlated to its acetone isoelectric point (Stearn and Stearn, 1924b). Gram-positive organisms have isoelectric points at low pH-values, in no case studied were they over a pH of 3 and mostly they were around a pH of 2. Gram-negative organisms have corresponding points at higher pH-values, usually about 5 to 5.5. There is also a fairly well defined class of organisms which has long been recognized as not having a pronounced Gram character. These have been considered by Hucker and Conn (1923 and 1927), by the present authors (Stearn and Stearn, 1925), and are referred to by Churchman as being "unstable Gram-positive bacteria" (1927). These organisms, of which *Bacillus diphtheriae* and *Bacillus dysenteriae* (Shiga) are examples, have isoelectric points at intermediate pH-values, usually between three and four.[2]

In the present study it was found that, as soon as chemically active decolorizers (*i.e.* distinctly acidic or basic) were employed, such a correlation no longer held. A glance at the curves shows that, tho the acetone isoelectric point of the Gram-positive Staphylococcus is at a distinctly lower pH-value than is that of the Gram-negative *Bacterium coli*, the anilin point for the former is at a higher pH-value than it is for the latter. The shifts due to acidic decolorizers in the

[2]It is not meant to indicate that Gram character is completely determined by isoelectric point. A preliminary discussion of the factors determining the Gram reaction has been given by the authors (Stearn and Stearn, 1924a and b), and a much more comprehensive treatment of the problem is soon to appear (Stearn and Stearn. University of Missouri Studies, 3, No. 2 (in press)).

two figures cannot be directly compared, since for the one the phenol curve is given while for the other the formaldehyde curve is given. Analysis of all our data, however, shows the same lack of correlation.

Justification for the apparently arbitrary assumption that the acetone isoelectric point is essentially the real chemical isoelectric point of the bacterium is found in the fact that the apparent shifts in this point due to the use of distinctly acidic decolorizers were, in all cases studied, in the direction of higher pH when referred to the acetone point, while the shifts due to the use of basic decolorizers were similarly always toward a lower pH-value. A piece of confirmatory evidence is the finding of Shaughnessy and Falk (1924) that the maximum buffering power of *Bacterium coli* occurs at a pH very close to the pH of the acetone isoelectric point.

An interesting feature of the curves is the behavior of the acidic decolorizers toward smears stained with the acidic dye, acid fuchsin. It will be noted that the retained color passes thru a maximum as the hydrogen-ion concentration increases. This effect is analogous to the effect, pointed out and discussed in the preceding paper in connection with a study of the effect of the chemical nature of the decolorizer on the results of the Gram reaction, of basic decolorizers on smears stained with basic dye. The present study brings out the symmetry of behavior of acidic decolorizers toward smears stained with acid dye. The effect met with in studying the Gram reaction on the part of the basic decolorizers and which is so well brought out in the curves for acidic decolorizers is not seen in these same curves for the basic decolorizers. The reason is that in this study the smears were taken only to a sufficiently high pH-value to give the isoelectric points. The limiting pH-value reached was usually not much over seven or at most eight, whereas free energy considerations indicate that, altho Lugol's solution has a pH-value not far from six, the mordant of Kopeloff and Cohen (1928), which was also used in the work reported in the former paper mentioned, is not far from pH 12.

It should be noted that this apparent anomaly, met with in the cases of both basic and acidic decolorizers and which has its explanation in the chemical nature of the action of these decolorizers, has never been met with by the authors when using acetone under similar conditions, and in the course of their work they have determined the isoelectric points of not far from fifty cultures, representing about sixteen different organisms.

We meet, then, with the following general behavior: Acidic decolorizers show a regular behavior toward smears stained with basic dye. They completely decolorize such smears up to pH-values higher than those at which the same dye is retained when acetone is used as

decolorizer. The mechanism is evidently a tendency to combine with the dye and remove it from bacterial combination. Toward smears stained with acid dye these same acidic decolorizers are often poorer decolorizers than acetone at ordinary pH-values, but at low pH-values, under conditions such that the bacteria are on the acid side of their chemical isoelectric points, these same acidic decolorizers tend to combine with bacteria, and thus, chemically competing with acid dye, their mass effect tends to replace the dye in combination. Analogously with basic decolorizers, their effect on acid dye-stained smears is regular, but the decolorizing of smears stained with basic dye may pass thru a maximum at high pH-values. Acetone has been found regular in its behavior toward smears stained with either type of dye. The dye retained by organisms increases regularly with pH if it be basic dye and increases regularly with hydrogen-ion concentration if it be acid dye.

As is pointed out above these results are easily explained on the basis of a chemical mechanism of staining, but the authors are unable to fit them into any so-called physical theory.

The purpose of the present paper is not to propose a specific universal decolorizer but to contribute to an attempt at rationalizing the problem of decolorization. There may be specific cases of differentiation where, for routine results, an acidic decolorizer would be more satisfactory than an inert one. It would perhaps be possible to work out conditions such that one could have a reliable technic for differentiating between the two Gram-negative organisms or two Gram-positive organisms by using proper decolorizing procedure. The development of such methods should be expedited by a knowledge of the characters which constitute important factors in the behavior of a substance as a decolorizing agent.

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CERTAIN FACTORS INFLUENCING THE STAINING PROPERTIES OF FLUORESCEIN DERIVATIVES

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Abstract.—**The writers discuss a series of investigations as to the behavior of certain fluorescein dyes (eosin, erythrosin, phloxine, and rose bengal) in staining bacteria in dried films of soil. These dyes are ordinarily purchased in the form of di-sodium salts and are indifferent staining agents for the purpose named. If there be added to the dye solution a small amount (0.001 to 0.1%) of a mineral salt of calcium, aluminium, magnesium or lead, the intensity of staining is greatly increased. The effect of such addition is to convert the dye partly into a salt of the metal added, which in nearly every instance is relatively insoluble and is in every case less soluble than the di-sodium salt. It is shown that practically identical results can be obtained if the staining be performed with a suspension of the calcium, aluminium or lead salt of one of these dyes, altho very little of the dye goes into solution.**

Theories to account for the phenomenon are discussed, including in particular the solution and adsorption theories of staining. The evidence seems to favor the former, altho not entirely disproving the latter.

A series of previous papers[1, 3, 4] has discussed the use of certain fluorescein derivatives (primarily rose bengal) in staining bacteria, particularly in soil. The technic is coming to be an important one for use in the study of soil bacteriology, having recently been adopted with some modifications by Winogradsky.[2] Unfortunately, however, not all who have tried the method have been successful in obtaining good preparations, and it seemed advisable to make an investigation of some of the factors that may influence results.

The original technic called for a phenolic solution of rose bengal. Winogradsky preferred a phenolic solution of erythrosin; and a later investigation by the writers[3] showed that this latter dye can, under certain conditions at least, give results as good as those obtained with

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[1]Conn, H. J. The microscopic study of bacteria and fungi in soil. N. Y. Agr. Exper. Sta., Tech. Bul. 64. 1918.

[2]Winogradsky, S. Études sur la microbiologie du sol: I Sur la méthode. Ann. Inst. Pasteur, 39, 299–254. 1925.

[3]Conn, H. J. and Holmes, W. C. Fluorescein dyes as bacterial stains. With special reference to their use for soil preparations. Stain Tech. 1, 87–95. 1926.

rose bengal. A later note by one of the writers[4] described an improved technic calling for erythrosin Y, phloxine B, or rose bengal in aqueous solution following pre-treatment with acetic acid. Altho stating then that good results can be obtained with all three dyes, none of them were found to give better results than rose bengal, and the writer's personal preference still remained for the latter stain. Reports from other soil bacteriologists, however, indicate that some of them, as well as Winogradsky, still obtain better results with erythrosin than with rose bengal.

Preliminary Observations

The first clue to the reason for these differences in results in different laboratories came thru the cooperation of one of the stain manufacturers. They had submitted to the Commission laboratory for approval a sample of rose bengal which did not give results as good as was desired. The company was surprised at the unfavorable report because the dye was identical chemically with the previous sample they had submitted and which had been found entirely satisfactory. To help find the trouble they made up nine solutions of this sample of the strength regularly employed (1%) and sent the solutions in to be tested, labelled by number only. Upon testing them it was found that one of them was better than any solution that had ever been used at Geneva, while the others were unsatisfactory, staining either too lightly or too intensely. Upon inquiry it was learned that four of the solutions were made up in varying strengths of malic acid, four others in varying strengths of lead acetate, while the one giving best results was a plain aqueous solution. This was disconcerting until it was learned that the company had used tap instead of distilled water in making their aqueous solution; and it was found upon investigation that the tap water at Geneva gave similar results. In other words, all that was necessary to do in order to obtain good results with this sample of rose bengal was to dissolve it in tap water instead of distilled.

This fact seemed to need further investigation. It was immediately suspected that the factor in the tap water which improved the results was the calcium. Accordingly solutions of this same sample of rose bengal were made up in distilled water containing different amounts of $CaCl_2$ or $CaSO_4$. The chloride was used in the following strengths: 0.001%, 0.01%, 0.03% and 0.1%; the sulfate was used in the strength of 0.001% and 0.01%, the latter being nearly saturated. It was quickly found that the solutions which contained only 0.001% of

[4]Conn, H. J. An improved stain for bacteria in soil. Stain Tech. 1, 125–8. 1926.

either salt stained only fairly, practically no better than the plain aqueous solution, while the 0.01% solutions gave good results. Best results of all were obtained with the solution containing 0.03% $CaCl_2$, while the one containing 0.1% of this latter salt was too intense, the dead organic matter as well as the bacteria staining deeply. The experiment was repeated with the same amounts of sodium chloride; but the staining results were no better than plain aqueous solutions.

It was noticed that those solutions which stained the best contained a very slight colored precipitate, and that by adding larger quantities of the calcium salt the amount of this precipitate could be greatly increased. The precipitate was assumed to be the calcium salt of rose bengal which is sparingly soluble in water. This precipitate was filtered off and staining attempted with the filtrate—without any better results than with the plain aqueous solution. If, however, the precipitate was removed from the filter paper and suspended in distilled water, and this suspension used in place of a staining solution, results could be obtained as good as with the solutions containing 0.01%–0.03% $CaCl_2$. In other words it was thus found that best results could be obtained, not with a clear solution, but with a suspension of a dye salt only sparingly soluble in water.

Theoretical

These results were of interest for two reasons: first, they suggested certain improvements in the technic of staining bacteria in soil which would make the procedure more reliable; secondly, they seemed to have some significance in explaining the method by which bacteria are stained by the acid dyes of this group. The further investigations were planned to shed light on both these points, theoretical as well as practical.

Before discussing the investigations carried on, a few words as to the theory of staining seem worth while. Briefly stated, the theories to explain staining phenomena are either chemical or physical. The chemical theories generally assume that the part of any tissue stained by a given dye is of opposite reaction from the dye ion; thus the acid nuclei take up basic dyes, and the basic cytoplasm the acid dyes. There seems little question but that many well-known staining phenomena can be explained on this theory. Physical theories of staining generally assume either that the dye is *ad*sorbed as a layer on the surface of the object colored by it, or that it is *ab*sorbed by the material of which this object is composed, passing in other words from solution in water to solution in the substance being stained. These theories are all complicated and are not so distinct from one another

as implied by the simple statement just given; but nevertheless one may say that roughly speaking there are three main theories of the action of stains on tissue: the chemical theory, the adsorption theory, and the solution theory.

Bacteria are ordinarily stained with basic dyes, and there seems little reason for questioning the applicability of the chemical theory in explaining the process. These dyes are difficult to wash out of bacteria stained with them, a fact which suggests chemical union; it has also been shown by the Stearns[5] that staining of bacteria with such dyes bears a relation to the isoelectric point of protoplasm which is best explained on a chemical basis. In the present procedure, however, acid dyes are used, which theoretically should not combine chemically with bacteria if the latter have a special chemical affinity for basic dyes. It is noticeable, moreover, that the dyes used in the present work do not cause intense staining and are very readily removed by too long washing. Such observations seem to point against the chemical theory in this instance; nor does this theory serve to explain the greater intensity of staining in the presence of small amounts of calcium.

For these reasons it seemed that the staining in the procedure under investigation must be a phenomenon of adsorption or of solution. If an adsorption phenomenon, the explanation of the action of calcium must be that in the presence of a readily adsorbed cation, the amount of an acid dye taken up is increased; this is indeed, a generally accepted fact. If a solution phenomenon, the explanation plainly is that the addition of a mineral salt of calcium converts the dye into its calcium salt which is less readily soluble in water than its sodium salt and hence able to pass more easily from aqueous solution into solution in the bacterial protoplasm.

To test this point five lines of investigation were undertaken: 1) sodium salts of various fluorescein derivatives were employed to observe whether their staining action is in inverse proportion to their solubility as would be called for on the solution theory; 2) dye salts of metals other than sodium were compared with each other in their staining action to see if there were any similar correlation between intensity of staining and solubility; 3) mineral salts of other metals than calcium were compared with the latter in their effect upon the staining action of dye solutions; 4) minute quantities of acid were added to solutions of the sodium salts of the dyes to convert the dyes into the less soluble mono-sodium salts and to observe the effect on

[5]Stearn, E. W. and Stearn, A. E. A study of the chemical differentiation of bacteria. J. Bact. 7, 529.

staining; 5) quantities of sodium chloride large enough to lower the solubility of a dye were added to a dye solution to notice whether the effect on staining were similar to that already observed with a small amount of the calcium salt.

It was felt that if a distinct relation between intensity of staining and solubility of the dye were observed in every instance there would be support of the solution theory. On the other hand it was realized that in some instances the results might be explained on the basis of selective adsorption; and as the latter theory has wide support among physical chemists who have studied dye action, it was considered that results favoring the solution theory must be accepted cautiously.

Experimental

Methods. Various solutions and suspensions of the dyes were employed in staining soil preparations. The technic used was as follows: Soil was mixed with 9 c.c. of a 0.015% solution of gelatin, and a small drop of the suspension spread into a thin film over a glass slide and dried on a flat surface, over a boiling water bath. Sometimes the slides were then dipped for two minutes into 40% acetic acid or strong HCl (1 part concentrated acid to 1 part water) followed by very brief washing; but in general they were stained without the acid treatment. In staining, the dye solution or suspension was placed on the soil film while the slide was lying on the flat surface of the water bath and allowed to stand for one minute, after which it was quickly washed off.

Tests with Various Salts of the Dyes. The first three lines of investigation indicated above were tested out simultaneously. For this work sodium salts of eosin Y, erythrosin, phloxine O (presumably CI No. 774) and rose bengal B (presumably CI No. 779) were obtained at the Color Laboratory of the Department of Agriculture, and were converted into other salts by the following method:

A concentrated aqueous solution containing a slight excess of the metallic salt over the quantity required to react with the dye was added to a concentrated aqueous solution of the dye; the precipitated metallic salt of the dye was then filtered off and washed with a small quantity of water.

These various salts, together with solutions of the sodium salts to which varying amounts of the corresponding mineral salts had been added were used in staining soil preparations.

The results are given in table 1. Before discussing the results it should be remarked that the writers' earlier work gave some indication that the sodium salts of fluorescein derivatives stain bacteria in soil films with increasing depth in inverse order to their solubility. It

was, in fact, found that of the four dyes, eosin Y, phloxine, erythrosin, and rose bengal, eosin stains the least, and rose bengal the deepest, while the other two are intermediate; and the solubility of these four dyes decreases in the same order. Further evidence on this point is obtained from a glance at the top row of results in table 1, altho in this instance poor results were obtained with phloxine and erythrosin as well as with eosin Y.

The results with the various salts of eosin Y are especially interesting, as the depth of staining in this case increases in the order of decreasing solubility. This can best be understood from the table by realizing that the salts are listed in order of solubility, the most soluble salts at the top, the least soluble at the bottom. With the other three dyes another factor enters in; for altho the magnesium and calcium salts stain more deeply than the sodium salts (sometimes too deeply, in fact) the results with the aluminium salts are weaker. This is undoubtedly to be explained by the fact that aluminium salts are entirely too insoluble to accomplish satisfactory staining.

The second part of the table showing the results with solutions of the sodium salts to which small amounts of mineral salts of magnesium, calcium, aluminium and lead have been added is an investigation of the third point named above (p. 97). The results seem to be very clear. On account of the greater solubility of eosin Y, it is necessary to add to it more of any one of these salts to secure good staining than with the other dyes, and best results are obtained with the use of lead acetate. With phloxine best results are also ordinarily obtained with the largest amounts of the mineral salts. It is particularly noticeable that no good results were obtained with the addition of $Mg\,SO_4$; this accords with the solution theory, for the magnesium salt of phloxine happens to be exceptionally soluble. With erythrosin and rose bengal the addition of any of these mineral salts increased the staining power, and in many instances the staining was too intense, especially in the case of rose bengal or the aluminium salts of erythrosin. Increasing the amount of the mineral salt always increased the depth of staining except in the case of lead acetate where the results were more irregular—a fact undoubtedly to be explained by the much greater insolubility of the lead salts of these dyes.

Another point to notice is the matter referred to in one of the footnotes, namely that whenever the precipitate was filtered off and the staining power of the filtrate tested, it was found that the bacteria stained no more deeply than with the sodium salt of the dye in question. This will be referred to again in the discussion below.

TABLE 1. RESULTS OF STAINING SOIL SMEARS WITH VARIOUS COMBINATIONS OF DYES AND SALTS (WITHOUT PRE-TREATMENT WITH ACID)

Dye Solution	Eosin Y	Phloxine	Erythrosin	Rose Bengal	
Na Salt	Poor. Too weak	Poor. Too weak	Poor. Too weak	Fair*. Too weak	
Mg Salt	Poor* " "		Fair† Sometimes slightly too intense. Ppt.	Good† Sometimes too intense	Four or five slides in each instance
Ca "	Poor* " "	Good Sometimes too intense	Fair to Good Sometimes slightly too intense. Ppt	Poor Too intense Ppt.	
Ba "	Poor* " "				Pre-treatment with 40% acetic acid tried in each case. Results as shown in foot-note.*
Al "	Fair* " "	Fair to Good†	Good	Fair to Good Sometimes too weak	
Pb "	Good*†				

Na salt made up in solution of $MgSO_4$					
0.1%	Poor. Too weak	Poor. Too weak	Fair to Good	Good { Almost too intense	
0.01%	Poor* " "	Poor " "	Fair to Poor { Too weak	Good	About 2 slides in each instance
0.001%	—	Poor " "	Poor " "	Good	
$CaCl_2$ 0.1%	Poor to Fair { " "	Good	Good	Good { Slightly too intense	
0.01%	Poor " "	Fair. Too weak	Fair. Too weak	Good " "	Pre-treatment with acetic acid tried only in those cases marked (*)
0.001%	—	Poor " "	Poor " "	Good	
$AlCl_3$ 0.1%	Poor to Fair { " "	Fair " "	Good { Sometimes too intense. Ppt.	Fair to Good { Sometimes too weak	2–6 slides in each instance
0.01%	Poor " "	Fair " "	Fair to Poor { Too weak	Good	
0.001%		Poor " "	Poor	Good { Sometimes too intense	
Lead Acetate 0.01%	Fair to Good {	Poor " "	Fair " "	Good	Only 1 slide each
0.001%		Fair " "	Good	Fair. Too weak	

*These instances were improved by pre-treatment with acetic acid.

†The staining power of these solutions was tested after filtering off precipitate. It was poor except in case of Mg salt of Rose Bengal, in which case it was about the same as the Na salt of this dye.

Effects of Adding Acid to the Dye Solutions. It was noticed earlier[6] that pre-treatment with 40% acetic acid improved the staining power of the sodium salts of the dyes, as did N/10 HCl. This action of the acid is presumably due to some of it remaining in the soil film and converting the di-sodium salt of the dye into the mono-sodium salt when the staining solution is applied. This is borne out by the fact that long washing after the acid treatment destroys its effects. It is found that HCl must be used in a much stronger solution than acetic in order to give as good results as the latter. Now considering the greater H-ion concentration of a solution of HCl than of one of acetic acid of corresponding dilution, this fact cannot well be explained except by assuming that the mineral acid is more easily washed out than the organic and hence has less effect upon the staining solution added subsequently.

TABLE 2. RESULTS OF STAINING SOIL SMEARS WITH ACIDIFIED DYE SOLUTIONS

	Erythrosin	Rose Bengal
Na Salt alone	Poor—almost unstained.	Fair to good—too weak
" " with 0.01% acetic acid	Good	Good (rather better than with $CaCl_2$)
" " with 0.1% acetic acid		Poor—too intense
" " with 0.01% $CaCl_2$	Fair—too weak	Good
" " with 0.001 N HCl	Fair—too weak	Good
" " with 0.002 N HCl		Good—slightly too intense
" " with 0.003 N HCl		Quite good—rather too intense
" " with 0.005 N HCl		Fair } Too intense, but neither so much so as the
" " with 0.01 N HCl		Fair } one with 0.1% acetic.

If this is the explanation of the action of the acid pre-treatment, the same results could be accomplished by adding very small amounts of the acid directly to the staining solution. This would convert the di-sodium salt into the less soluble mono-sodium salt and therefore increase its staining power. This point was investigated and the results are given in table 2 in the case of rose bengal. It will be seen that the addition of 0.01% acetic acid gives good staining, while with the addition of 0.1% the action is too intense. With HCl good staining is obtained in a 0.001N solution, while a solution of only 0.002N gives too intense staining. This solution is much weaker than the 0.01% acetic acid solution, if figured on the basis of the actual amount of acid present, but is perhaps about the same in H-ion

[6]See footnote 4.

concentration. The difference between the relative strengths of these two acids needed when used this way and that when used for pre-treatment suggests that the reason why acetic acid works better in the case of pre-treatment is, as suspected, because it washes off less easily. This seems to present further evidence that the staining of bacteria with these dyes is dependent upon the solubility of the dye.

Effect of Adding Sodium Chloride. To test out the last point mentioned on p. 97–8 there was added to a 1% solution of rose bengal enough NaCl to cause the dye to become saturated and to begin to precipitate. This of course required a large amount of the salt. This solution was then used for staining by the usual method. Good results were obtained, much better than without the large addition of the salt.

This observation, like the others which had been made, indicates an inverse relation between dye solubility and intensity of staining in the technic under investigation.

Discussion

These results are interesting both from the standpoint of theory and from that of practical staining, in that they show an apparent relation between staining and solubility of the dye. The facts observed cannot be explained by the chemical theory and it is questionable whether the adsorption theory accounts for them in all instances. On the other hand, the solution theory of dyeing apparently affords a simple and adequate explanation of all the observations. It is very probable that both factors are operative, altho the present investigation supports the view that this particular staining reaction is primarily a solution phenomenon. Investigations are under way, however, which may throw further light on the matter, and the writers' views on the theoretical aspect of the procedure are open to subsequent revision.

Regardless of which theory to employ to explain the results, it is evident that to secure most intense staining of bacteria in soil with one of these dyes, one should have a solution of a dye which is not only saturated but contains a slight excess of the dye. If this excess is great, the staining is likely to be so intense that the results are not satisfactory for the purpose of staining bacteria in soil where one does not want the dead organic matter to be colored.

It must be distinctly understood that these observations and the possible theories to explain them apply only to the staining of bacteria with dyes of the fluorescein group. No data are at hand yet to show whether similar factors operate in regard to the staining of bacteria

with other dyes or in regard to the staining of other kinds of tissue with fluorescein derivatives.

These observations bear on practical staining problems in various ways. In the first place they suggest a reason why different samples of rose bengal differ in their ability to stain bacteria in soil. The accidental presence of 0.1% of some calcium, magnesium, or aluminium salt in a sample of rose bengal or erythrosin would be enough to give a concentration of 0.001% in a 1% solution of the dye and would thus assure good staining results, while a more carefully purified sample of the same dye might give less satisfactory results. It also seems likely that a sample of the dye too pure to give good results in staining some soils might work well with others containing a larger amount of calcium, or that a sample giving good results with the first soil might stain the other too deeply. Such considerations as these undoubtedly explain why some soil bacteriologists obtain better results with rose bengal, others with erythrosin.

For all these reasons, altho one might perhaps obtain best results using the magnesium or calcium salt of one of these dyes, it does not seem well to recommend that they be purchased in that form. The best plan seems to be to purchase either rose bengal or erythrosin as the sodium salt, just as at present, and then for each bacteriologist who is using the technic to determine what concentration of some mineral salt of calcium, magnesium, or aluminium to add to give staining effects of the right depth with his soil.

Technic Recommended

Accordingly the following technic is now recommended: Prepare soil infusion as previously described, one gram of soil to 9 c. c. of 0.015% gelatin solution. Dry on a microscopic slide, preferably over a boiling water bath. Stain with a 1% solution of erythrosin or rose bengal in a solution containing between 0.001% and 0.1% $CaCl_2$. The exact amount of $CaCl_2$ to employ is very important and should be determined in advance for the dye sample used and the soil to be stained; one should use just enough to give deep staining of the bacteria without too intense coloration of the dead organic matter. Usually such a solution will contain a very faint precipitate of the calcium salt of the dye, which must not be removed but should be brought into suspension by shaking when the staining solution is used. The staining solution should remain in contact with the soil film for about one minute, while resting on a level surface over a boiling water bath; and then should be washed off rapidly. The preparation is then ready for examination under a microscope.